THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.
110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985



Application for Research Grant
(Use extra pages as needed)

Date: June 21, 1973

1. Principal Investigator (give title and degrees):

John R. Daniels, M.D.
Assistant Professor of Medicine

2. Institution & address:

Stanford University
Stanford, California 94305

3. Department(s) where research will be done or collaboration provided:

Department of Medicine
Department of Radiology
Department of Pathology

4. Short title of study:

Interstitial Fibrosis of the Lung. Development of a Mouse Model System



5. Proposed starting date: January 1, 1974

6. Estimated time to complete: Three (3) years

7. Brief description of specific research aims:

The specific goals of this study may be summarized as follows:

1. Characterization and comparison of lung collagens and the collagen of fibrosis
   a. Isolation by chromatography
   b. Cyanogen bromide peptide maps and amino acid analysis
2. Evaluation of therapeutic intervention in lung fibrosis
   a. Lathrogen (βAPN)
   b. Corticosteroid
3. Refinement of the model of lung fibrosis following radiation injury
   a. Improvement of reproducibility by attention to bacteriologic control
   b. Development of a reliable and sensitive biochemical measure of pathologic fibrosis
   c. Detailed correlation of morphologic, biochemical and functional alterations

8. Brief statement of working hypothesis:

It is proposed to study a model of pulmonary radiation fibrosis in mice. Observed structural changes will be correlated with measurements of functional capacity and detailed biochemical analysis. These correlative studies should provide the background for systematically exploring therapeutic manipulations. Initial experimental questions include the following:

1) The time course and radiation dose relationship for the development of fibrosis will be determined.
2) Biochemical measures of total collagen content will be assessed and correlated with the pathologic appearance.
3) Pulmonary function (measures of compliance) will be obtained and an attempt made to correlate microscopic, functional and biochemical changes.

The therapeutic effects of modifications of collagen synthesis will be explored looking at survivorship, morphology, compliance and the biochemical expression of fibrosis. A biochemical, structural description of lung collagen will be undertaken and compared with the collagen involved during pathologic fibrosis.

9. Details of experimental design and procedures (append extra pages as necessary)

See appended pages

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Personal laboratory space is 400 square feet of standard design. It is generally well equipped and well stocked. Common equipment facilities and controlled temperature rooms are in adjacent space. Personal and common equipment capabilities include extensive column chromatographic equipment, spectrophotometer, recorder, pH meter, flash evaporator, $CO_2$ incubator, laminar flow hood, preparative and analytical ultracentrifuges, scintillation counters, amino acid analyser, high voltage electrophoresis, acrylamide gel electrophoresis, sonifier and a variety of partition chromatographic apparatus. Animals will be housed (as noted in Methods) in a new barrier facility under strict bacteriological control.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (oppend):

See appended pages

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See appended pages

14. First year budget:

| A. Salaries (give names or state "to be recruited") | % time | Amount |
|---|---|---|
| Professional (give % time of investigator(s) even if no salary requested) | | |
| DANIELS, John R. - Principal Investigator | 35 | |
| BROWN, John Martin - Co-Investigator | 10 | |
| FAJARDO, Luis F. - Co-Investigator | 2 | |
| Technical | | |
| To be recruited - Research Technician | 100 | |
| To be recruited - Dishwasher | 20 | |
| To be recruited - Secretary | 20 | |
| FRINGE BENEFITS | | |
| Sub-Total for A | | |

B. Consumable supplies (by major categories)

| | |
|---|---|
| Animals ($4,500. See attached Budget Justification) | |
| Chemicals | |
| Glassware | |
| Disposables | |
| Chromatographic Supplies | |
| Sub-Total for B | $ 7,000. |

C. Other expenses (itemize)

| | |
|---|---|
| Maintenance Contracts | 600. |
| Travel | 1,000. |
| Publication Costs | 300. |
| Histologic Preparations | 1,000. |
| Sub-Total for C | $ 2,900. |
| Running Total of A + B + C | $34,971. |

D. Permanent equipment (itemize)

| | |
|---|---|
| Amino Acid Analyser Automation | 5,000. |
| UV Monitor with 310 mμ capability | 3,000. |
| Fraction Collector | 1,000. |
| Recorder | 700. |
| Constant Temperature Circulator | 700. |
| Pump (2) | 600. |
| Sub-Total for D | $11,000. |
| E | 5,245. |
| Total request | $51,216. |

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|---|---|---|---|---|---|---|
| Year 2 | REDACTED | $7,000. | $3,100. | 0 | $5,523. | $42,344. |
| Year 3 | | $7,500. | $3,300. | 0 | $5,884. | $45,112. |

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| Mammalian Collagenase and Basement Membrane Collagen | NIH #AM 14896-03 | $26,177. | 2/1/73 - 1/31/74 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| Cancer and Bone Metabolism | American Cancer Society | $132,903. | 1/1/74 - 12/31/76 |
| Laboratory Studies: Support of Clinical Cancer Center (Program Project Grant Application) | NIH/NCI | $700,245. | 1/1/74 - 12/31/76 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name John R. Daniels

Signature John R Daniels Date 6/21/73

Telephone 415 (Area Code) 321-1200 (Number) 5878 (Extension)

Checks payable to

Kenneth D. Creighton
Deputy Vice President for Business and Finance

Mailing address for checks

Stanford University

Stanford, California 94305

Responsible officer of institution

Typed Name Kathleen C. Butler

Title Sponsored Projects Officer

Signature Kathleen C. Butler Date 6/29/73

Telephone (415) 321-2300 Ext 2883 (Area Code) (Number) (Extension)

100539531

## Budget Justification

### Personnel

Salary increases of 5.5% per year are calculated.

In accordance with University policy, salary support for Dr. Daniels is requested at a fractional level commensurate with anticipated effort. No support is requested for Drs. Brown, Fajardo.

A research technician is budgeted to assist in irradiation of animals, preparation of tissues and all of the biochemical studies. A part-time equivalent secretary is budgeted to purchase supplies, keep books and type correspondence and manuscripts related to this proposal. A part-time equivalent dishwasher is budgeted for washing glassware.

### Capital Equipment

Chromatographic equipment. Biochemical characterization of collagen (see Methods of Study) will involve extensive column chromatography of peptides requiring monitoring in the far UV (310 mμ to 330 mμ). The funds will cover purchase of spectrophotometer, flow cells, recorder, pump, fraction collector and constant temperature circulator.

Amino acid analyser automation. Upgrading of an existing analyser will accomodate the increased utilization involved in column determination of hydroxyproline. Specific changes include automated multiple sample applicator, programming for unattended operation and recording of digitalizad data to allow computer processing.

### Supplies

The cost of animals is a function of the large number which we anticipate using as well as the cost of maintaining barrier conditions for bacteriologic control.

# INTERSTITIAL FIBROSIS OF THE LUNG. DEVELOPMENT OF A MOUSE MODEL SYSTEM.

## Introduction

The characteristic pathologic expression of several human disease states results from unfavorable proliferation of connective tissue (Rhodin, 1967). In such cases scarring of the organ rather than parenchymal cell destruction results in diminished functional capacity. There are several examples of this in human parenchymal disease, but among the most common clinically is interstitial pulmonary fibrosis (Spencer, 1968). Similar alterations in other organs include contractures of skin following chemical or thermal destruction (Peacock et al., 1968), the immobilization by fibrotic adhesions of reconstituted tendons, esophogeal stricture following chemical irritation (lye ingestion or gastric reflux) (Davis, 1971), and hepatic cirrhosis with portal hypertension (Orloff et al., 1967; Rubin and Hutter, 1967). Interstitial fibrosis of the lung results from limited injury which may be caused by infection (bacterial, viral, mycoplasma, richettsia), dusts, chemical irritation, circulatory abnormalities, radiation and perhaps immunologic mechanisms (Spencer, 1968). Fibrosis may follow from several different sites of primary injury: 1) organization of intra-alveolar exudates as seen following lobar pneumonia; 2) alveolar epithelial damage as seen following accretion of dust within macrophages, penetration of alveolar epithelium with fine dusts or as a consequence of hypersensitivity reactions; 3) alveolar capillary damage as seen in sclerosing alveolitis, rheumatoid disease, systemic sclerosis and radiation injury (see below); and 4) chronic lymphedema as seen in mitral stenosis.

Fibrosis as a late manifestation of the response to radiation injury is readily apparent in several tissues and may be the dominant mode of toxicity. Among those tissues in which these radiation induced alterations are of clinical importance are lung (Bennett et al., 1969), myocardium, pericardium (Steward et al., 1967; Fajardo, 1970) and bowel.

Pulmonary radiation fibrosis is readily produced in experimental animals (Jennings and Arden, 1961; Jennings and Arden, 1962). It thus serves both as a specific model for the commonly encoutered clinical problem of radiation injury and as a general model to study pulmonary fibrosis and by extension the problem of pathologic fibrosis in all tissues. Work to date has primarily been descriptive. We propose to extend these studies by correlating observed structural changes with measurements of functional capacity and detailed biochemical analysis. These correlative studies should provide the background for systematically

exploring potential therapeutic manipulations. Several direct experimental questions will be proposed initially: 1) the time course and radiation dose relationship for the development of fibrosis will be determined; 2) biochemical measures of total collagen content will be assessed and correlated with the pathologic appearance; 3) pulmonary function (measures of compliance) will be obtained and an attempt made to correlate microscopic, functional and biochemical changes. The therapeutic effects of modifications of collagen synthesis will be explored looking at survivorship, morphology, compliance and the biochemical expression of fibrosis. A biochemical, structural description of lung collagen will be undertaken and compared with the collagen involved during pathologic fibrosis.

## Background

### Pulmonary Morphology Following Radiation Injury

Clinical recognition of pulmonary injury by therapeutic radiation dates to 1898 (Bergonie and Tessier, 1898). Early reports emphasized compromised pulmonary function, increased densities on chest X-ray and fibrosis on autopsy specimens (Hines, 1922). In a review of material obtained from an NIH Cooperative Lung Study (Bennett et al., 1969), in 6 of 72 examined specimens the patients were thought to have died with radiation pneumonitis as the immediate or major contributing cause of death. No single pathognomonic morphologic feature was identified by examination by light microscopy. The diagnosis was made from a combination of alveolar septal thickening, proliferation and desquamation of atypical septal cells, hyaline membrane formation and pulmonary vascular changes. Alveolar cell hyperplasia with bizarre cytopathy was perceived as the most specific change for radiation injury. Alveolar septal thickening initially consisted of edema and mononuclear infiltrate which then evolved to fibrillar thickening and eventually to dense collagenization. Small vessel alteration has included capillary obliteration, thickening of arterial walls and fragmentation of elastica in larger vessels.

In general, these alterations have been reproduced in experimentally irradiated rodents (Jennings and Arden, 1961, 1962) but refined understanding of the precise sequence of events has been hampered by several clinical and experimental difficulties: 1) limitations of resolution of light microscope; 2) superimposed infection (Kurohara and Casarett, 1972); 3) species variation; 4) influence of damage of remote tissues in whole body radiation (as suggested by experiments with parabiots [Goldenberg et al., 1968]); 5) influence of

uneven ventilation and variations in $O_2$ tension; and 6) in humans, underlying coexisting pulmonary disease.

Elegant electron micrographic studies have more recently emphasized primary vascular endothelial injury (Phillips, 1966; Phillips, 1973; Adamson et al., 1970). The description that follows is derived primarily from Adamson et al.: Within the first week after 600 rad total body irradiation, vacuolation of capillary endothelial cells occurs with balooning of cytoplasm into the capillary lumen. Subendothelial swelling separates the endothelial cell from the supporting basement membrane. The epithelium of Type I squamous and Type II cells with lamellar bodies remains relatively unaffected. The endothelial vacuolation so stretches the cytoplasm that capillary lumen become occluded, and by ten days the basement membrane may be completely stripped of the overlying endothelium. Platelet thrombi attach to the denuded basement membrane by two weeks. At this time minor alterations in epithelial cells may be appreciated. Fibrin is deposited within the platelet thrombus, and organization with fibroblasts and subsequent deposition of collagen follows.

Biochemistry of Fibrosis. Collagen Chemistry and Crosslink Formation

Research concerning the biosynthesis of scar has centered on collagen metabolism since it is the principal extracellular protein synthesized during active fibrosis and since collagen fibers are the predominant material in scar (Ramachandran, 1967). The maturation of collagen involves the progressive loss of solubility by extensive crosslink formation. It is appropriate to review fundamental collagen chemistry and the mechanism of crosslink formation (Traub and Piez, 1971; Gallop et al., 1972).

The molecule is an asymmetric rigid rod with dimensions of 15 Å x 3000 Å. Each molecule consists of three polypeptide chains each of which contains approximately 1,000 amino acid residues. Throughout the course of most of the molecule, polypeptide strands are arranged in the unique collagen helix pattern. In each polypeptide chain every third residue is glycine. These glycyl residues occupy the axial plane. Individual collagen molecules form fibrils by precipitating in a linear quarter-stagger array. Fibril formation proceeds spontaneously in the extracellular space under physiologic conditions. Crosslink formation is an important element in fibril stabilization. Crosslinks exist within each molecule between the polypeptide chains in the non-helical amino terminal portion. Intermolecular crosslinks within the fibril are much more extensive but somewhat less well understood. These crosslinks link many single molecules into a large polymer. The detailed chemistry of collagen

crosslinking has been well studied in organs such as skin and bone. The ε-amino group of specific lysyl residues, predominantly those in the amino-terminal region, undergo enzymatic oxidative deamination (allysine oxidase) to the ε-amino adipic-δ-semialdehyde. Crosslinks are formed when modified residues on adjacent chains undergo aldol condensation reactions or when Schiff base formation takes place between a modified lysyl or hydroxylysyl residue and one which has not been modified or with another amine. These Schiff bases become stabilized by subsequent reduction. More recently a greater variety of additional spontaneous condensations of aldehydes has been identified. The products of these condensations have been isolated from highly crosslinked collagen polymers and suggest the involvement within one complex crosslink of three lysines and one histidine from four separate polypeptide strands. These crosslinks may occur in the helical portion of the molecule, but their precise location is as yet unknown. The key initial reaction for the formation of all crosslinks, both intra- and inter-molecular, involves the initial oxidative deamination of the ε-amino group of lysine. This enzyme, allysine oxidase, may be selectively inhibited by lathrogens. Thus crosslink formation can be blocked without affecting collagen synthesis, collagen secretion or spontaneous fibril formation. The paradigm compound β-aminopropionitrile (βAPN) has been demonstrated in vitro specifically to inhibit the enzyme allysine oxidase (Pinnell and Martin, 1968; Siegel et al., 1970; Siegel and Martin, 1973). Chronic exposure of animals during growth to this compound results in connective tissue of low tensile strength and high solubility.

Biochemical Modification of Scar Formation

Because of the central importance of collagen synthesis and crosslinking in fibrosis, scar formation and wound healing, there is wide interest in investigating compounds which may potentially manipulate collagen metabolism. The most extensively studied method of altering collagen is the use of lathrogens such as βAPN, and attempts have been made to use lathrogens to ameliorate the functional alteration associated with fibrosis in experimental animals and in human disease. Models in which early evidence of probable effectiveness have been obtained include silica-induced pulmonary fibrosis (Leveue and Bye, 1964), carbon tetrachloride-induced hepatic fibrosis (Fiume, 1962), and lye-induced esophogeal stricture (Davis, 1971). Pilot clinical studies have not been as rewarding. The hide-bound skin of scleroderma has not been improved by the use of lathrogens (Keiser and Sjoerdsma, 1967), and while increased range of

motion may be affected by lathrogens following tendon repair, this beneficial effect has been lost when the drug is stopped (Peacock et al., 1968).

A second method for the inhibition of collagen crosslinking has utilized compounds which inhibit crosslinking by complexing with the aldehydes that are formed from allysine oxidase. D-Penicillamine is the model compound which has been used clinically (Harris and Sjoerdsma, 1966). Patients with systemic sclerosis have been treated with relatively disappointing results. They may have slightly increased mobility of skin by special testing, but the progression of disease is unaffected (Fulghum and Katz, 1968).

A more recent major investigational pathway is the use of proline analogs such as azetidine-2-carboxylic acid, 3-4-dehydroproline, cis-hydroxyproline and 4,5-dehydrolysine (Lane et al., 1971; Rosenbloom and Prockop, 1970). Effects on collagen synthesis with all these compounds have been recently investigated. The first two of these compounds are incorporated into the collagen molecule on the order of four residues per 1,000. They apparently inhibit extrusion of the collagen molecule from the cell so that formation of fibrils is diminished. Some experiments indicate that these compounds decrease granuloma formation by as much as 50% when tested in the carrageenin granuloma model.

The potential role of corticosteroids in the management of radiation lung injury is as yet unresolved. In patients acute pneumonitis may respond to corticosteroids (Cosgriff and Kligerman, 1951; Friedenberg and Rubenfeld, 1954; Ruben et al., 1958), and flares of acute radiation pneumonitis on occasion appear to be recalled following withdrawal of corticosteroids which have been incidentally administered (personal observation). It is unclear, however, whether corticosteroids will affect the development of fibrosis. Studies with animal models have been inconclusive (Brown, 1956) to a large extent because of infection (Stewart, personal communication).

Biochemistry of Lung Collagen

Very little published information exists which specifically concerns lung collagen despite its obvious importance both in the normal structure of the lung and in the diseases in which a fibrosis reaction is prominant. Anatomic considerations lead to the prediction of at least three kinds of collagen: 1) collagen fibrils contribute to the interstitial matrix; 2) collagen of the cartilaginous type (Miller, 1972) is present in the bronchial skeleton; 3) the alveolar and capillary basement membrane may also be predominantly a collagen with a unique structural gene analogous to basement membrane of renal glomerulus and

and lens capsule (Kefalides and Denduchis, 1969). None of these collagens has been individually, cleanly isolated, and no primary structural data is as yet available. In addition, it is not known whether the collagen seen in a pathologic fibrosis reaction shares the structural gene of the involved organ. There are no studies which effectively deal with spontaneous rates of collagen turnover in the lung, and none which quantify collagen synthesis during periods of active fibrosis in the lung.

The specific goals of this study may be summarized as follows:

1. Characterization and comparison of lung collagens and the collagen of fibrosis
   - a. Isolation by chromatography
   - b. Cyanogen bromide peptide maps and amino acid analysis
2. Evaluation of therapeutic intervention in lung fibrosis
   - a. Lathrogen (βAPN)
   - b. Corticosteroid
3. Refinement of the model of lung fibrosis following radiation injury
   - a. Improvement of reproducibility by attention to bacteriologic control
   - b. Development of a reliable and sensitive biochemical measure of pathologic fibrosis
   - c. Detailed correlation of morphologic, biochemical and functional alterations

Methods to be utilized include:

1. Techniques

Radiation (supervised by Dr. J. Martin Brown)

Anesthetized mice (sodium pentobarbital, 67.5 mg/kg IP) will be placed in a jig which allows irradiation of six aminals simultaneously. The radiation field will be right hemithorax alone or entire thorax with midline shielding to protect the esophagus. Conditions will be 250 kv, 15 ma, focused skin distance 40.5 cm, added filtration of 0.25 mm Cu and 1.0 mm Al, HVL 1.1 mm Cu and a dose rate of 157 rads. All measurements are made with a Philips dosimeter calibrated against a standard Victoreen chamber (NBS calibrated). A factor of 0.95 is used to convert exposure in roentgens to rads in tissue.

Bacteriologic Control and Animal Selection (supervised by Dr. J. Martin Brown)

The animals used in these experiments will be specific pathogen free (SPF). In several reported studies (Brown, 1956; Kurohara and Casarett, 1973) as well as in our own preliminary work, microscopic changes are characterized by great

variation among animals in both acute inflammatory changes as well as degree and distribution of fibrosis. It appears than an important variable may be infection. Our preliminary studies have shown the $LD_{50/160}$ for thorax-irradiated conventional mice to be approximately 1400 rads single dose. The comparable statistics elsewhere (Yuhas, 1973) is twice this dose when SPF mice are utilized. We are in the process of building up stocks of SPF mice (C3H/Km, Balb/C, and C56BL/Ka). The procedure involves caesarian delivery under sterile conditions and foster nursing on SPF mice as described (Serrano, 1971). Strict barrier conditions will be maintained for breeders. Animals used in experiments will be kept under less rigid conditions in cages with filter tops (Maryland Plastics, New York). All operations such as drug injections and irradiation will be performed under sterile conditions in filtered, laminar flow air.

Microscopic Evaluation (supervised by Dr. Luis Fajardo)

Dose/response curves for pathologic changes in morphology will be performed on unilaterally irradiated mice to avoid the systematic selection of minimally affected animals which results from studying survivors of whole thorax irradiation. Mice will be treated at age 4 to 6 months. In initial studies mice will be sacrificed by decapitation to minimize anesthetic induced changes. Lungs will be fixed in 10% buffered formalin and sections 4-6 μ thick will be stained by hematoxylin and eosin as well as Gamori's trichrome method for connective tissue. In initial experiments the dose delivered will range from 600 to 3600 rads in a single exposure, and animals will be sacrificed at 2, 4, 8, 16, and 32 weeks. Scoring will include assessment of presence and distribution of both inflammatory changes (granulocytes, histiocytes, lymphocytes, fibrin) and fibrosis. Conditions of bacteriologic control and irradiation will be varied to minimize infection and also death by radiation damage to other organs such as esophogus, heart and pericardium.



Biochemical Measures of Degree of Fibrosis (supervised by Dr. John Daniels)

Two approaches to the biochemical quantification of the collagen of lung fibrosis will be pursued:

a) Entire lungs from unilaterally irradiated mice will be separated, hydrolysed (6 N HCl, anaerobic), and hydroxyproline content determined by Dowex 50 separation and ninhydrin reaction on an automatic amino acid analyser. Preliminary studies have shown this method feasible with a standard deviation of about 10%.

b) Highly specific collagenase will be prepared by gel permeation chromatography and selective inhibition with N-ethyl maleimide (Peterkofsky and Diegelmann,

1971). Powders of irradiated lungs will be prepared in a Wiley mill, washed in neutral 20% NaCl and 0.5 M acetic acid and incubated with collagenase. Solubilized material will be hydrolysed and amino acid hydrolysis performed both to quantitate material released and to determine, independent of hydroxyproline, that it is collagen. Since the degree of hydroxylation of collagen is an unpredictable variable, this second method will independently assess the validity of the easier hydroxyproline determinations.

Characterization of Lung Collagen

Lung collagen of mature mice (age 4 to 6 months) will be solubilized by limited proteolysis with pepsin (Kefalides and Denduchis, 1969; Miller, 1972; Daniels,-1973). In parallel studies weanling mice will be raised on a lathyritic diet (see below) and lungs sequentially extracted with 1 M NaCl, pH 7.4 and 0.5 M acetic acid. The collagen will be purified by salt precipitation (Bornstein and Piez, 1966). After denaturation, monomers (α-chains) will be selected by gel permeation chromatography (agarose, BioGel A 5) and separated by ion exchange chromatography sequentially on columns of carboxymethyl cellulose and diethylaminoethyl cellulose. Isolated α-chains will be characterized by amino acid analysis and by peptide maps developed after limited hydrolysis at methionyl residues by cyanogen bromide (CNBr) (Bornstein and Piez, 1965; Butler et al., 1967).

The collagen of the fibrosis reaction will be obtained by placing adult mice on lathyritic diets immediately after maximally effective hemithoracic irradiation and isolating collagen without pepsin digestion. Under these conditions we would expect to be able to extract only the collagen synthesized after exposure to radiation (while on βAPN) and thus separate the collagen of the fibrosis reaction from that pre-existing in normal lung. Collagen thus obtained will be characterized as before (chromatography, amino acid analysis and peptide maps) and compared with fractions isolated from normal lung.

Administration of βAPN

All mice will be maintained on Purina Cat Chow. This feed is in the form of a star-shaped kernel with a high surface volume ratio. βAPN will be dissolved in water and sprayed on the kernels (2 grams βAPN/kg food) which are air-dried. Feasibility studies have confirmed that this effectively produces lathyrism in growing C3H mice and is without apparent toxicity over a six-month period for adult (4 to 6 months old) mice.

Measurement of Pulmonary Compliance

It is expected that the fibrosis reaction will be associated with decreased compliance. Mice to be studied will be sacrificed by $CO_2$ intoxication. The trachea will be percutaneously canulated with a plastic catheter which is secrued by a ligature. After intubation the thoracic cage is dissected free by removing overlying skin and transecting at the level of the neck and the diaphragm. The diaphragm is removed. In feasibility studies, we were able to obtain such preparations consistently and without air leaks from both normal animals and mice after irradiation. Compliance is measured by connecting the cannula to a pressure transducer and to a calibrated syringe. Air is added or withdrawn in 0.1 ml increments, and the intrapulmonary pressure is electronically recorded.

2. Summary of Anticipated Sequence of Experiments

a. Group 1. Dose/response for morphologic change with hemithoracic irradiation.
Dose/response for survival. Bilateral irradiation. Establishment of $LD_{50/160}$ in SPF C3H mice.

b. Group 2. Methodology for biochemical measure. Comparison of time-dependent change between irradiated and shielded hemithorax and selection of method (hydroxyproline vs. collagenase release).
Characterization of collagen of fibrosis reaction (hemithoracic irradiation and lathyritic diet).
Compliance studies in sublethally, bilaterally irradiated animals.

c. Group 3. Correlative studies: morphologic, biochemical and mechanical changes as a function of time and dose after bilateral irradiation.
Effects of βAPN on correlative studies and on $LD_{50/160}$ days.



General Significance of Proposed Study

This injury model if successfully developed will have a general utility in future studies. Studies of other effectors of lung injury including the entire range of chemical inhalents could be extended to utilize the biochemical and functional techniques explored here. A model will be established which evaluates the effects of therapies in functional and biochemical as well as morphologic dimensions. In short, the model system may provide a general experimental

tool for diverse problems in the etiology and treatment of pulmonary fibrosis.

Lung collagen will be characterized in the way other collagens have been characterized, and the general question of whether the structural gene for fibrosis is the same as the gene for interstitial collagen of the affected organ will be approached.

The specific question of usefulness of lathrogens in ameliorating the functional disturbances secondary to scar formation in radiation injury of the lung will be answered. This may have important implications for this same question in other tissues and following other injuring agents.

References


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## Facilities Available

Personal laboratory space is 400 square feet of standard design. It is generally well equipped and well stocked. Common equipment facilities and controlled temperature rooms are in adjacent space. Personal and common equipment capabilities include extensive column chromatographic equipment, spectrophotometer, recorder, pH meter, flash evaporator, $CO_2$ incubator, laminar flow hood, preparative and analytical ultracentrifuges, scintillation counters, amino acid analyser, high voltage electrophoresis, acrylamide gel electrophoresis, sonifier and a variety of partition chromatographic apparatus. Animals will be housed (as noted in Methods) in a new barrier facility under strict bacteriological control.

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Biographical Sketch of Principal Investigator

John R. Daniels

Date and Place of Birth:

REDACTED

Education:

R A.B. Stanford University
M.D. Stanford University

Research and/or Professional Experience

| | |
|---|---|
| 1972- | Assistant Professor, Department of Medicine, Division of Oncology, Stanford University School of Medicine |
| 1970-72 | Instructor, Department of Medicine, Division of Oncology, Stanford University School of Medicine |
| 1969-70 | Senior Resident, Department of Medicine, Stanford University School of Medicine |
| 1966-69 | Research Associate, National Institute for Dental Research, National Institutes of Health, Bethesda, Maryland |
| 1965-66 | Assistant Resident, Department of Medicine, Stanford University School of Medicine |
| 1964-65 | Intern, Department of Medicine, Stanford University School of Medicine |
| 1964 | Post-doctoral Fellow, Department of Cell Biology, Albert Einstein College of Medicine, New York |
| 1959-62 | Trainee, Department of Pharmacology, Stanford University School of Medicine |

Major Investigative Interest:

Connective Tissue Chemistry; Clinical Oncology

Honors:

Diplomate, American Board of Internal Medicine, 1972

Research Support

Current Support:
Mammalian Collagenase and Basement Membrane Collagen, NIH #AM 14896-03. February 1, 1973 - January 31, 1974. $26,117. 50% effort.

Pending applications:
Cancer and Bone Metabolism, American Cancer Society. January 1, 1974 - December 31, 1976. $132,903. 15% effort.

This proposal is also being submitted to the NIH as part of a Program Project Grant entitled Laboratory Studies: Support of Clinical Cancer Center. January 1, 1974 - December 31, 1976. $700,245. 23% effort.

Publications

Kalman, S.M., and J.R. Daniels, Effect of injected estradiol on the uptake of α-aminoisobutyric acid by tissues of the ovariectomized rat. Biochem. Pharm. 8:250, 1961.

Publications (continued)

Daniels, J.R., and S.M. Kalman, Effects of estradiol on water uptake and α-aminoisobutyric acid accumulation by uteri of hepatectomized rats. J. Endocrin. 28:73, 1963.

Lazarus, G.S., J.L. Decker, C.H. Oliver, J.R. Daniels, C.V. Multz, and H.M. Fulmer, Collagenolytic activity of synovium in rheumatoid arthritis. New Eng. J. Med. 279:914, 1968.

Lazarus, G.S., R.S. Brown, J.R. Daniels, and H.M. Fulmer, Human granulocyte collagenase. Science 159:1483, 1968.

Lazarus, G.S., J.R. Daniels, R.S. Brown, H.A. Bladen, and H.M. Fulmer, Degradation of collagen by a human granulocyte collagenolytic system. J. Clin. Invest. 47:2622, 1968.

Barrow, M.V., L.F. Mills, J.R. Daniels, and J. Lian, The use of acrylamide gel in immobilizing Alizarin-stained skeletal preparations. Stain Tech. 44: 162, 1969.

Lazarus, G.S., J.R. Daniels, J. Lian, and M.C. Burleigh, Role of granulocyte collagenase in collagen degradation. Am. J. Path. 68:565, 1972.

Daniels, J.R., J.M. Lian, and G.S. Lazarus, Human granulocyte collagenase. J. Biol. Chem., in press.

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Biographical Sketch of Co-Investigator

John Martin Brown

Date and Place of Birth:

REDACTED

Education:

B.Sc. Birmingham University, Birmingham, England
M.Sc. London University, London
D.Phil. Oxford University, Oxford

Research and/or Professional Experience:

1971- Assistant Professor, Department of Radiology, Radiobiology Research Division, Stanford University School of Medicine
1970-71 Research Associate, Department of Radiology, Radiobiology Research Division, Stanford University School of Medicine
1968-70 Post-doctoral Fellow, Department of Radiology, Radiobiology Research Division, Stanford University School of Medicine

Major Investigative Interest:

Mammalian cell, tissue and tumor radiobiology, cell kinetics, radiation carcinogenesis

Honors:

Medical Research Council Scholarship, Oxford University, 1965-68
NIH Fellowship in Radiobiology and Cancer Research, 1968-70
American Cancer Society (California Division) Dernham Senior Fellowship in Oncology, 1971-74

Research Support:

Current Support:

Dernham Senior Fellowship in Oncology, #D-188, American Cancer Society (California Division). September 1, 1972 - August 31, 1973. $16,400. Salary support only.

Training Program in Cancer Research in Radiology, NIH #CA-05008, NIH-NCI. July 1, 1972 - June 30, 1976. $311,990. 15% salary support.

Pending support:

CORE Grand, NIH #CA-10372-07, NIH-NCI. September 1, 1973 - August 31, 1974 $436,771. 10% salary support.

Dr. Brown is also named on the Program Project Grant of this project referred to on Dr. Daniels' Biographical Sketch. 10% salary support.

Publications:

Brown, J.M., and R.J. Berry, The relationship between diurnal variation of the number of cells in mitosis and of the number of cells synthesizing DNA in the epithelium of hamster cheek pouch. Cell Tissue Kinet. 1:23, 1968.

Brown, J.M., and R.J. Oliver, A new method of estimating the cell cycle time in epithelial tissues of long generation time. Cell Tissue Kinet. 1:11, 1968.

Publications (continued)

Brown, J.M., and R.J. Berry, Effects of X-irradiation on cell proliferation in normal epithelium and in tumors of the hamster cheek pouch. In Effects of Radiation on Cellular Prolferation and Differentiation, IAEA Symposium, pp. 475-491, 1968.

Brown, J.M., Long $G_1$ or $G_0$ state: A method of resolving the dilemma for the cell cycle of an in vivo population. Exp. Cell Res. 52:565, 1968.

Hall, E.J., J.M. Brown, and J. Cavanagh, Radiosensitivity and the oxygen effect measured at different phases of the mitotic cycle using synchronously dividing cells of the root meristen of Vicia faba. Rad. Res. 35:622, 1968.

Brown, J M., and R.J. Berry, A study of the effect of X-irradiation on the cell population kinetics in a model tumour and normal tissue system and the implications to the treatment of human malignancies. Brit. J. Radiol. 42:372, 1969.

Brown, J.M., and F. Ellis, The use of pyrimidine analogues in radiotherapy. Brit. J. Radiol. 42:155, 1969.

Brown, J.M., The effect of X-irradiation on the cell proliferation of induced carcinomas and their normal counterpart. Rad. Res. 43:627, 1970.

Brown, J.M., and D. Goffinet, A technique for intra-arterial infusion of tumor-bearing mice. J. Lab. Clin. Med. 76:175, 1970.

Brown, J.M., D.R. Goffinet, J.E. Cleaver, and R.F. Kallman, Preferential radiosensitization of mouse sarcoma relative to normal skin by chronic intra-arterial infusion of halogenated pyrimidine analogs. J. Nat. Cancer Inst. 47:75, 1971.

Goffinet, D.R., J.M. Brown, M.A. Bagshaw, and H.S. Kaplan, Prolonged carotid arterial radiosensitizer infusion and radiation therapy of mouse gliomas. Amer. J. Roentgen. 114:7, 1972.

Biographical Sketch of Co-Investigator

Luis F. Fajardo

Date and Place of Birth:

REDACTED

Education:

REDACTED A.B. Liceo de la Salle, Bogota, Colombia
M.D. Universidad Nacional, Bogota, Colombia

Research and/or Professional Experience:

| | |
|---|---|
| 1972- | Associate Professor, Department of Pathology, Stanford University School of Medicine |
| 1966-72 | Assistant Professor, Department of Pathology, Stanford University School of Medicine |
| 1965- | Pathologist, Veterans Administration Hospital, Palo Alto, California |
| 1962-65 | Chief, Department of Pathology, Universidad Nacional, Bogota, Colombia |
| 1960-62 | Assistant Professor, Department of Pathology, Universidad Nacional, Bogota, Colombia |
| 1959-60 | Pathologist, New Britain General Hospital, New Britain, Connecticut |

Major Investigative Interest:

Radiation pathology

Honors:

Founder, First Volunteer Blood Bank in Colombia, 1961
Founding Member and Secretary-Treasurer (1963-65), Colombian Society of Pathology



Research Support:

Current Support:
Sequential Study of Ultrastructure Changes in Experimental Radiation Heart Disease, MRIS #2735-01, VA Research Funds. July 1, 1972 - June 30, 1975 $68,400. 25% effort.

Publications

Cohn, K.E., J.R. Stewart, L.F. Fajardo, and W. Hancock, Heart disease following radiation. Medicine 48:281, 1967.

Stewart, J.R., K.E. Cohn, L.F. Fajardo, W. Hancock, and H.S. Kaplan, Radiation-induced heart disease. A study of twenty-five patients. Radiol. 89:302, 1967.

Stewart, J.R., L.F. Fajardo, K.E. Cohn, and V. Page, Experimental radiation-induced heart disease in rabbits. Radiol. 91:814, 1968.

Fajardo, L.F., J.R. Stewart, and K.E. Cohn, Morphology of radiation-induced heart disease. Arch. Path. 86:512, 1968.

Fajardo, L.F., Editorial (by invitation), Morphology of radiation-induced heart disease. J.A.M.A. 206:2309, 1968.

Fajardo, L.F., and J.R. Stewart, Experimental radiation-induced heart disease. I. Light microscopic studies. Am. J. Path. 59:299, 1970.

Publications (continued)

Stewart, J.R., and L.F. Fajardo, Dose response in human and experimental radiation-induced heart disease. Application of NSD concept. Radiol. 99:405, 1971.

Stewart, J.R., and L.F. Fajardo, Radiation-induced heart disease. Clinical and experimental aspects. Radiol. Clin. North Amer. 9:511, 1971.

Fajardo, L.F., and J.R. Stewart, Capillary injury preceding radiation-induced myocardial fibrosis. Radiol. 101:429, 1971.

Stewart, J.R., and L.F. Fajardo, Radiation-induced heart disease. In J. Vaeth (ed.) Frontiers of Radiation Therapy and Oncology, Vol. 6, S. Karger AG, Basel, pp. 274-288, 1972.

Fajardo, L.F., and H. Sarasti, Technique for percutaneous needle biopsy of bone and marrow. Calif. Med. 177:21, 1972.

Rockwell, S.C., R. Kallman, and L.F. Fajardo, Characteristics of a serially transplanted mouse mammary tumor and its tissue culture adapted derivative. J. Nat. Cancer Inst. 49:735, 1972.